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Award Number: DAMD17-03-1-0031

TITLE: Analysis of the Role of the Wnt/B-catenin Pathway in Prostate Development and Tumorigenesis

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REPORT DATE: April 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2006	Final	1 APR 2003 - 31 MAR 2006

4. TITLE AND SUBTITLE
Analysis of the Role of the Wnt/B-catenin Pathway in Prostate Development and Tumorigenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0031

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Van Andel Research Institute
Grand Rapids, MI 49503

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

14. ABSTRACT

We have continued studies which are focused on understanding how dysregulation of the Wnt/B-catenin signaling pathway are causally associated with prostate tumorigenesis. We have created a mouse model in which B-catenin signaling is activated and found that these mice develop prostate tumors with 100% penetrance. This process initiates with small areas of prostatic hyperplasia as early as 4.5 weeks of age, continues on to lesions resembling prostatic intraepithelial neoplasia (PIN), and progresses to invasive prostate carcinoma by 7 months of age. We have examined these mice at older ages and found that these tumors do not appear to metastasize. In addition, we have found that these tumors are initially androgen sensitive, based on the apoptotic response of these tumors to surgical castration, however, mice examined five months after castration reveal small areas of hyperplasia occurring in an androgen-independent manner. Finally, we are embarking on studies to determine if activation of B-catenin signaling can synergize with other genetic lesions in prostate cancer progression.

15. SUBJECT TERMS
Prostate cancer, Wnt, B-catenin, Apc, metastasis, PIN, hyperplasia

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	21	**19b. TELEPHONE NUMBER** *(include area code)*

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4. Introduction/Overview

To determine the role of dysregulated Wnt signaling in the development and progression of prostate cancer, we have successfully generated several mouse models. We have made the most progress using the cre-lox system to generate mice lacking the *Apc* gene in the prostate. *Apc* normally controls the level of β-catenin protein in the cytoplasm of cells. Loss of *Apc* leads to increased levels of the β-catenin protein and subsequent activation of downstream signaling pathways. We have found that mice lacking *Apc* in the prostate develop early onset prostate hyperplasia that is visible as early as four weeks of age. The tumors then show progression to full prostate carcinoma within 4 months, and show invasion into the surrounding stroma by seven months. We aged a cohort of 25 animals to between twelve and fifteen months of age and found that all 25 mice had advanced prostate tumors that displayed evidence of invasion into the local stromal tissue. However, despite the presence of enlarged lymph nodes suggestive of an inflammatory reaction, we do not see any evidence of metastasis to distant organs. We are in the process of writing this work up for submission to a peer reviewed journal. We anticipate submitted this work to the journal *Cancer Research* by May 19, 2006.

5. Body

Rationale (taken from original grant application)

Prostate cancer causes over 40,000 deaths per year in the United States (1). Most deaths are due to the metastatic spread of prostate cancer throughout the body. Currently, the only effective therapy for advanced prostate cancer is androgen depletion by surgical or chemical castration. This often causes temporary remission of the tumor. Unfortunately, prostate cancer commonly recurs in these patients in a form that is androgen-independent. There is currently no effective treatment for androgen independent prostate cancer and there is an urgent need to develop effective therapies for this disease.

β-catenin is a protein that plays multiple roles in regulating cell growth and function (2). Normally, the cytoplasmic level and nuclear localization of β-catenin is tightly regulated. In many tumors, however, this regulation is lost, either due directly to mutations in the *β-catenin* gene or by mutations in genes whose protein products are necessary for this regulatory process (2). One example is colon cancer, where the vast majority of tumors display increased cytoplasmic levels and nuclear localization of β-catenin due to loss of the *APC* gene (3). Over 20% of advanced prostate tumors have elevated levels of β-catenin, and mutations in the *β-catenin* gene have been identified in prostate tumors (4, 5). β-catenin can specifically associate with the androgen receptor (AR) (6-8). This interaction alters the signaling capabilities of the AR, making it more promiscuous in its ability to be activated by steroid hormones other than androgens (9). Based on these observations, β-catenin activation represents is a viable target for therapeutic intervention in advanced prostate cancers.

Objective/Hypothesis. The hypothesis underlying this proposal is that activation of β-catenin signaling contributes to the progression of prostate cancer to a malignant state**.**
Specific Aims We will directly test the effects of activated B-catenin on **1.)** prostate development and homeostasis and **2**.) progression of prostate cancer in a mouse model that normally develops prostate hyperplasia and dysplasia**.**

Study Design. We have created and analyzed trangenic mouse strains that overexpress β-catenin in the prostate epithelium. We have systematically analyzed these mice at various ages at the anatomical and histological level for abnormalities in prostate development and histology. We are assisted in these experiments via collaboration with Dr. Wade Bushman. Dr. Bushman has extensive experience in the analysis of mouse models of prostate development and tumorigenesis (12-15), and this proposal represents the continuation of an established collaborative relationship between our laboratories.
In support of this work, a post-doctoral fellow in my laboratory has traveled to Madison, Wisconsin to spend time in the Bushman laboratory to learn more about techniques in prostate analysis. This fellow, Dr. Troy Giambernardi, devotes 50% of his effort towards this project and is funded by a grant from the American Cancer Society. A full time technician, Holli Charbonneau, will begin has worked on this project since April of 2004. Holli had previously worked on this project as an undergraduate intern from April 2003-March 2004. Dr. Giambernardi left my laboratory to take a position as a research scientist

in another laboratory at our Institute in July 2005. Ms. Charbonneau left to start her studies in medical school in July 2005 as well. Katia Bruxvoort, a research technician, started work on this project in August 2005 and made excellent progress in finishing the work relevant to this grant until the end of the granting period in March 2006.

Objective 1: To determine the effect of transgenic activation of β-catenin on prostate morphology.

Task 1. Develop a plasmid construct that directs the expression of an activated form of β-catenin under the control of the ARR2PB promoter (ARR2PB-S37A β-catenin), sequence confirm, and prepare for microinjection (Months 1-2)

Task 2. Perform pronuclear microinjection (in collaboration with Bryn Eagleson) and screen resulting offspring for the presence of the transgene (Months 3-5)

Task 3. Generate offspring from each founder line to establish strains (Months 6-10)

Progress:

Mice carrying a transgene directing the expression of an activated form of B-catenin under the control of the modified probasin promoter (ARR2PB) (10, 16) were created by pronuclear microinjection. This was performed by Bryn Eagleson, director of the VARI Transgenic Core Facility. Twelve potential founders were created and nine of those transmitted the transgene through the germline. We have established breeding lines for each of these and have begun to screen the males in each line for proper expression. This work has been delayed recently because the room that these lines were being maintained in was exposed to mice that arrived from the NCI-Frederick mouse facilities that carried mouse hepatitis virus (MHV) (17). These mice were sent to numerous facilities throughout the country. Luckily, our vivarium is a shower-in; barrier facility in which each of the cages is maintained in an isolated environment. Our vivarium staff tested every cage and found that the MHV infection was contained within two cages in that room. We made the decision to sacrifice most of the animals in the room and maintain a small number of cages in an isolated room so that we could rederive the strains back into our facility in a clean manner. We have done this for three of the lines (partly based on the initial screening of these lines described below). We have now re-initated the work using these strains.

Task 4. Screen males from founder lines for proper expression of activated β-catenin (Months 11-14)

We have collected samples from the nine lines for analysis. We have performed Western analysis on lysates from these lines. Our preliminary analysis suggested that at least two of the lines, 1655 and 1764, expressed the TetON protein at high levels in the dorsal and ventral lobes of the prostate. Initial analysis of the other lines suggested that they did not express the proteins to the same level. We have also collected samples for immunohistochemical analysis. We have not been able to use the antibody we used for

Western analysis to detect the TetON protein in formalin-fixed paraffin sections. We are in the process of evaluating in situ hybridization based approaches for cell-specific expression of the TetON protein.

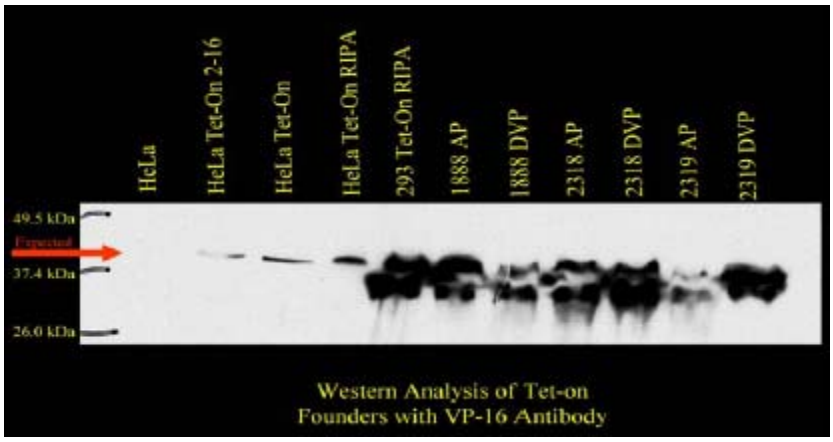

Figure 1.Mice carrying a transgene directing the expression of a the Tet-ON protein express the TetON protein in the prostate. Samples from a cell line not expressing TetON (HeLa), cell liens expressing the TetON protein (HeLa TetON), and tissue lysates from various transgenic mice were obtained. (AP=anterior prostate or coagulating gland; DVP=pooled Dorsal-ventral glands) 1888, 2318, and 2319 were three independent transgenic mice from the 1764 line.

We have also begun to evaluate the functionality of the two best strains, 1764 and 1655, by crossing these strains to strains which express various reporter genes under the control of the tetracycline responsive element (TRE). Compound trangenic mice, upon exposure to doxycycline either in the drinking water or the food, should express the gene under the control of the TRE. We have obtained a strain of mice that expresses both the Wnt1 oncogene and the luciferase reporter (18). This strain was generously provided by Dr. Lewis Chodosh (University of Pennsylvania). We have crossed this strain with the 1764 strain and generated compound transgenic male mice. We have exposed these mice to doxycycline for three months and then taken samples of the prostatic lobes. Our initial analysis suggested that this does not induce significant changes in the prostate epithelial. We have also initated crosses to the 1655 strain and will collect samples from them for analysis to confirm our results.

In addition to crossing the 1764 strain to the Wnt1-luciferase strain, we have also crossed it to a strain that expresses an oncogenic version of K-ras. We chose this because we had access to this strain at the beginning of the year and knew the strain worked in other contexts (for example, modeling lung cancer (19)). Induction of K-ras in compound transgenic mice resulted in prostatic dysplasia. This further supports the functionality of the 1764 strain for prostate cancer modeling in the mouse.

Task 5. Generate increased numbers of mice for analysis (Months 14-19)

Task 6. Analyze mice at the desired ages for prostate morphology at the gross anatomical and histological level (Months 20-36)

Progress:

We have begun to cross the 1764 strain to a strain of mice that expresses an activated, oncogenic version of B-catenin (S37A B-catenin). Analysis of this mouse strain in other contexts revealed that B-catenin could be induced with doxycycline treatment. We are generating the relevant mice on which to evaluate the effect of oncogenic B-catenin on prostate growth. We have generated compound transgenic mice for the 1764 strain and the TRE-Bcatenin strain. Our analysis has not shown any detectable phenotypic abnormalities, although we have only looked at mice in which B-catenin was ectopically expressed for less than four months.

We have also pursued an alternative approach to alter Wnt signaling specifically in the prostate. This was done by creating mice in which the Apc gene was specifically inactivated in prostate tissue via the use of the cre/lox system. In this approach, mice are genetically engineered which contain genes in which genetic regions required for function are flanked by *loxP* sites or "floxed." These floxed alleles retain normal function until they are exposed to cre recombinase. Upon exposure to cre, the genetic elements between the loxP sites are excised, leading to inactivation of the specific gene (20). We have created mice carrying a floxed allele of *Apc* via generation of chimeric mice (and subsequent breeding) using embryonic stem cells obtained from Dr. Tetsuo Noda (Japan) (21). Mice expressing cre in a prostate specific manner (*Probasin-cre* or *PB-cre*) were obtained from Dr. Pradip Roy-Burman (University of Southern California) (22). Through several rounds of mating, we have created mice that are homozygous for the floxed allele of *Apc* and also carry the *PB-cre* transgene (*PB-cre;Apc-flox/flox*).

Our analysis has shown that such mice enlarged develop prostatic hyperplasia as early as four and a half weeks of age (Table 1). Since loss of the Apc gene is associated with increased levels of the β-catenin protein, we confirmed that these lesions observed in young animals were associated with increased β-catenin levels (Figure 2).

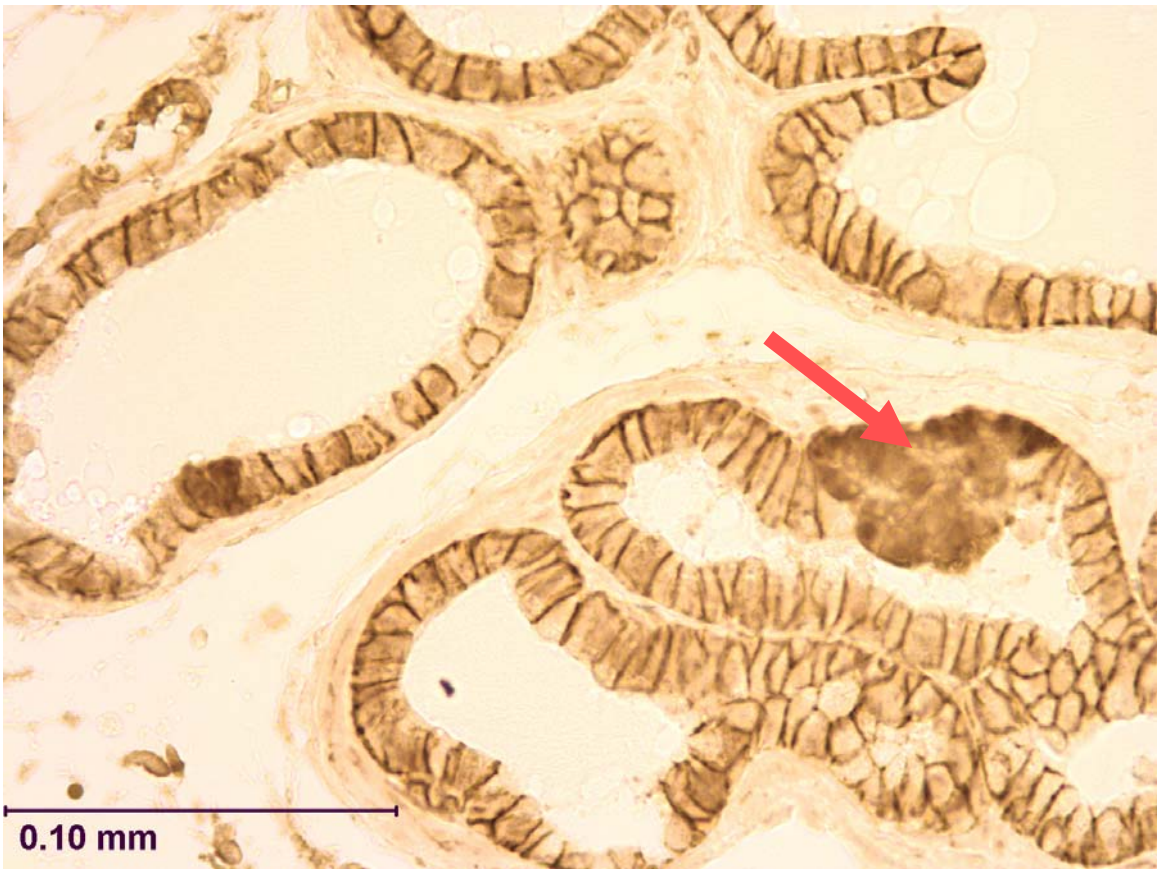

Figure 2. Early evidence of prostate hyperplasia in *PB-cre;Apc-flox/flox* mice. Sections of prostate tissue from a seven week old male were stained for the β-catenin protein. Note areas of increased β-catenin staining are associated with abnormal prostate architecture (red arrow)

We have allowed a cohort of such animals to age and found via analysis of animals at 4 and 7 months of age that these mice continue to progress to more aggressive states of hyperplasia displaying widespread prostatic intraepithelial neoplasia (PIN) and areas of microinvasion (Figure 3). We have also noted the presence of enlarged lymph nodes in these animals, but find no evidence for metastatic cells in these areas (Figure 4).

Table 1. Summary of Phenotypes Seen in *PB-cre;Apc-flox/flox* mice.

4.5 Weeks	**7 Weeks**	**3 Months**	**7 Months**	**11 Months**
Hyperplasia & small amount of PIN developing	PIN & stromal reaction (thickening and edema)	PIN & squamous metaplasia	Small areas of locally invasive adenocarcinoma	More extensive invasive adenocarcinoma

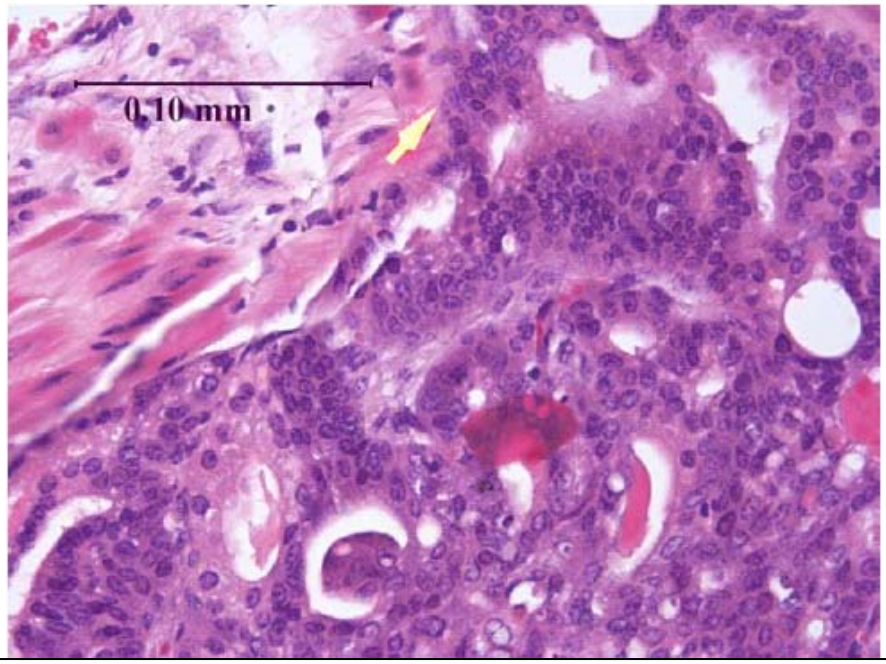

Figure 3. Prostate tumors in *PB-cre;Apc-flox/flox* mice are locally invasive by seven months of age. Shown is a tumor in which areas of invasion into the normal stroma (arrow) are shown.

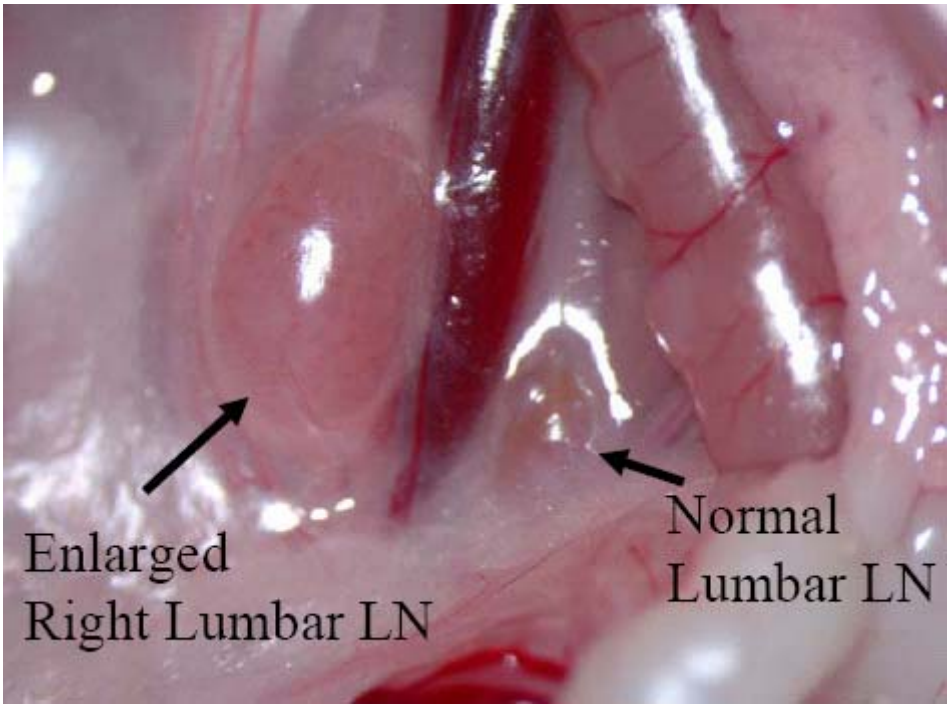

Figure 4. *PB-cre;Apc-flox/flox* mice develop enlarged lymph nodes associated with the development of prostate tumors. Shown is a gross dissection of the lumbar lymph node region. Note the obvious enlargement of the right lumbar lymph node (LN).

We have also examined whether androgen deprivation (via surgical castration) can inhibit the development of these tumors and whether the tumors ever become androgen independent. Analysis showed that castration lead to an immediate regression of these tumors associated with an induction of apoptosis (Figure 5). We aged cohorts of these mice to determine if tumors return after a latency period in an androgen-independent state. Interestingly, we found that 4-5 months after castration, evidence of small lesions in the prostate were found in these castrated PB-cre;Apc-flox/flox mice suggesting that this model was capable of at least early stages of androgen-independent prostate tumor growth (Figure 6).

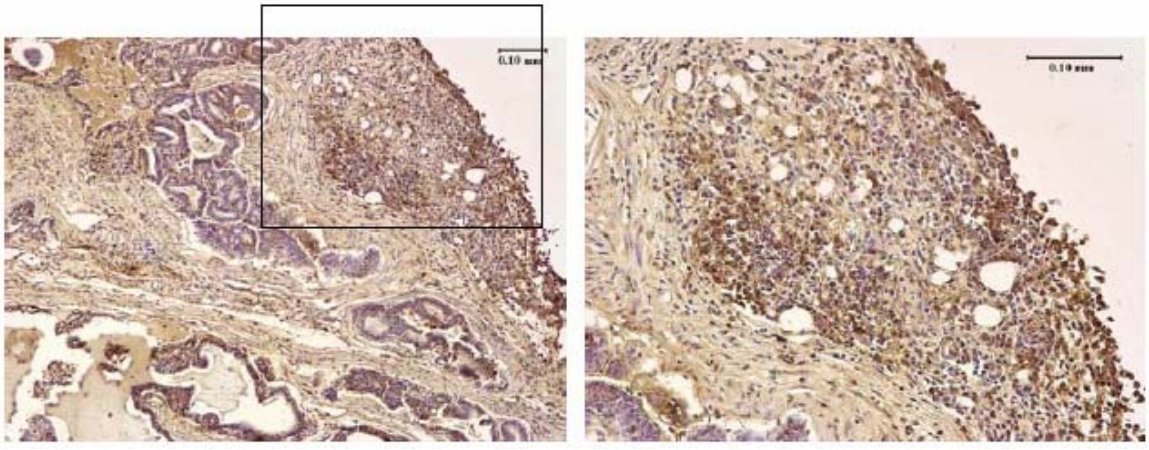

Figure 5. Prostate tumors in *PB-cre*;*Apc-flox/flox* mice initially remain androgen responsive. Shown is TUNEL staining to detect the presence of apoptotic cells (dark brown staining). In all cases examined, the tumors regress upon surgical castration. The image on the right is a high powered view of the region indicated by the box on the left (six days post-castration).

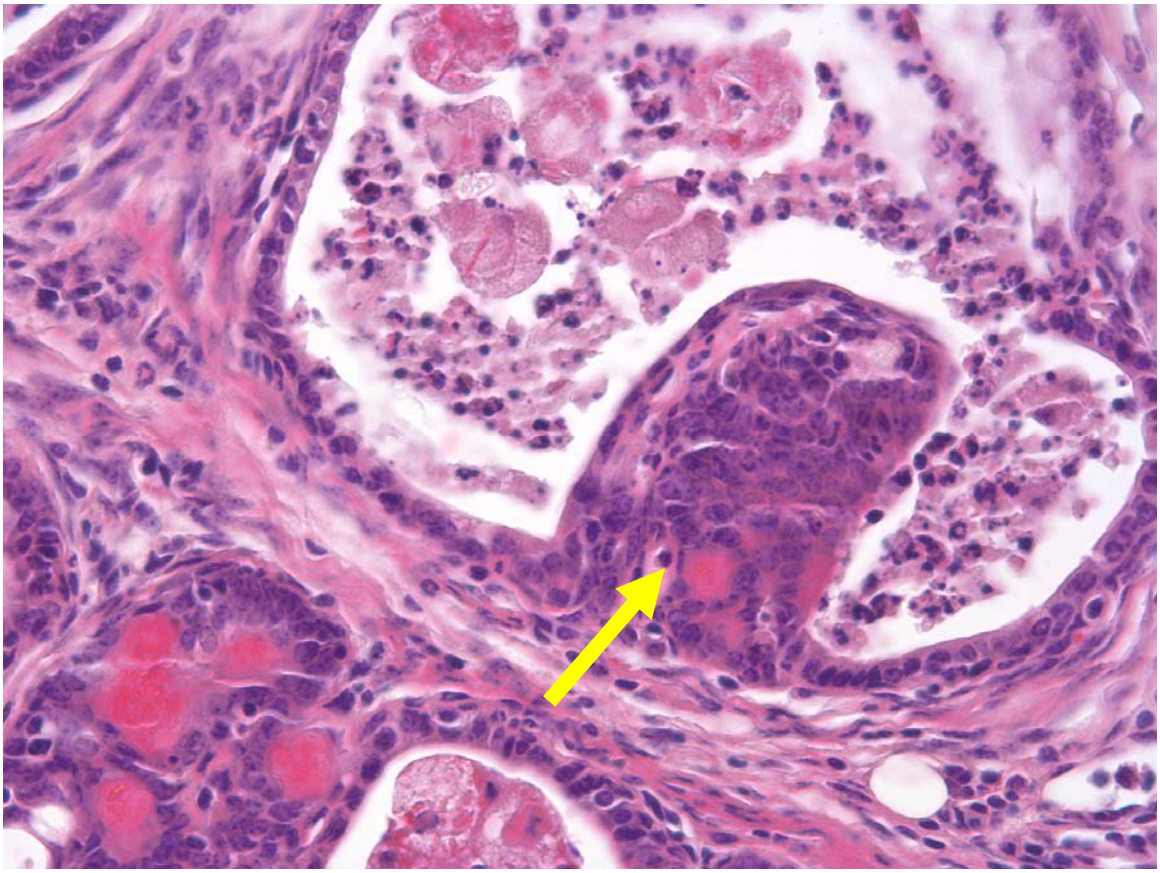

Figure 6. Androgen-independent prostate hyperplasia in castrated *PB-cre;Apc-flox/flox* mice. Note the polyp-like region (yellow arrow) in the prostate of this animal castrated 5 months prior to sacrifice and analysis.

Objective 2: To examine the effect of transgenic activation of β-catenin on inducing prostate cancer in the *Nkx3.1*-deficient mouse.

Task 1. Order Nkx3.1-deficient mice from the Mouse Models of Human Cancer Consortium repository and establish a colony in the Van Andel Institute mouse facility. (Sometime within the first 12-14 months)

Task 2. After identifying which ARR2PB-S37A β-catenin transgenic strains exhibit the desired expression patterns of β-catenin (Objective 1, Task 4), breed these strain(s) to the Nkx3.1-deficient mice. It will require two generations of crosses to generate ARR2PB-S37A β-catenin transgenic mice with varying Nkx3.1 genetic status. (Months 14-19).

Task 3. Analyze mice at the desired ages for prostate morphology at the gross anatomical and histological level (Months 20-36)

We have obtained *Nkx3.1*-deficient mice (11) from the MMHCC repository in Frederick, Maryland and successfully rederived them into our barrier facility at VARI. We have crossed them with the *PB-cre;Apc-flox/flox* mice to generate mice carrying a prostate-specific deletion of Apc that are also deficient for *Nkx3.1*. We will evaluate whether dysregulation of these two pathways results in synergistic effects on prostate tumor progression and metastasis. Finally, we are also generating mice that are deficient for both the *Apc* and *Pten* genes in the prostate by creating mice of the following genotype: *PB-cre;Apc-flox/flox;Pten-flox/flox*. Given that prostate-specific deletion of *Pten* leads to prostate cancer in the mouse that metastasizes to the lung (23), and that our work on this grant has shown that mice lacking *Apc* also develop prostate cancer, we are interested in determining whether there may be a synergistic effect of loss of these two genes. Given the role of Wnt signaling in bone development (24), we are especially keen to determine whether a mouse model of prostate cancer that metastasizes to the bone can be developed in any of these contexts. We have some initial analysis that suggests a dramatic enhancement of tumorigenesis in such mice lacking both *Apc* and *Pten* in the prostate epithelium (Figure 7). We are currently generating cohorts of mice to confirm the synergism in tumorigenesis caused by deletion of both genes.

Towards the end of this project we also generated preliminary evidence that *PB-cre;Apc-flox/flox* mice which also carry an activatable allele of *K-ras* (28) specifically in the prostate develop early onset metastatic prostate carcinoma. For the purposes of this report, we sacrificed one of our oldest mice of this genotype and found what appeared to be prostate tumor metastasis in the lungs and liver of these mice (Figure 8). We are very excited about this result and hope it will help form the basis of a competitive funding proposal for a grant from the National Cancer Institute.

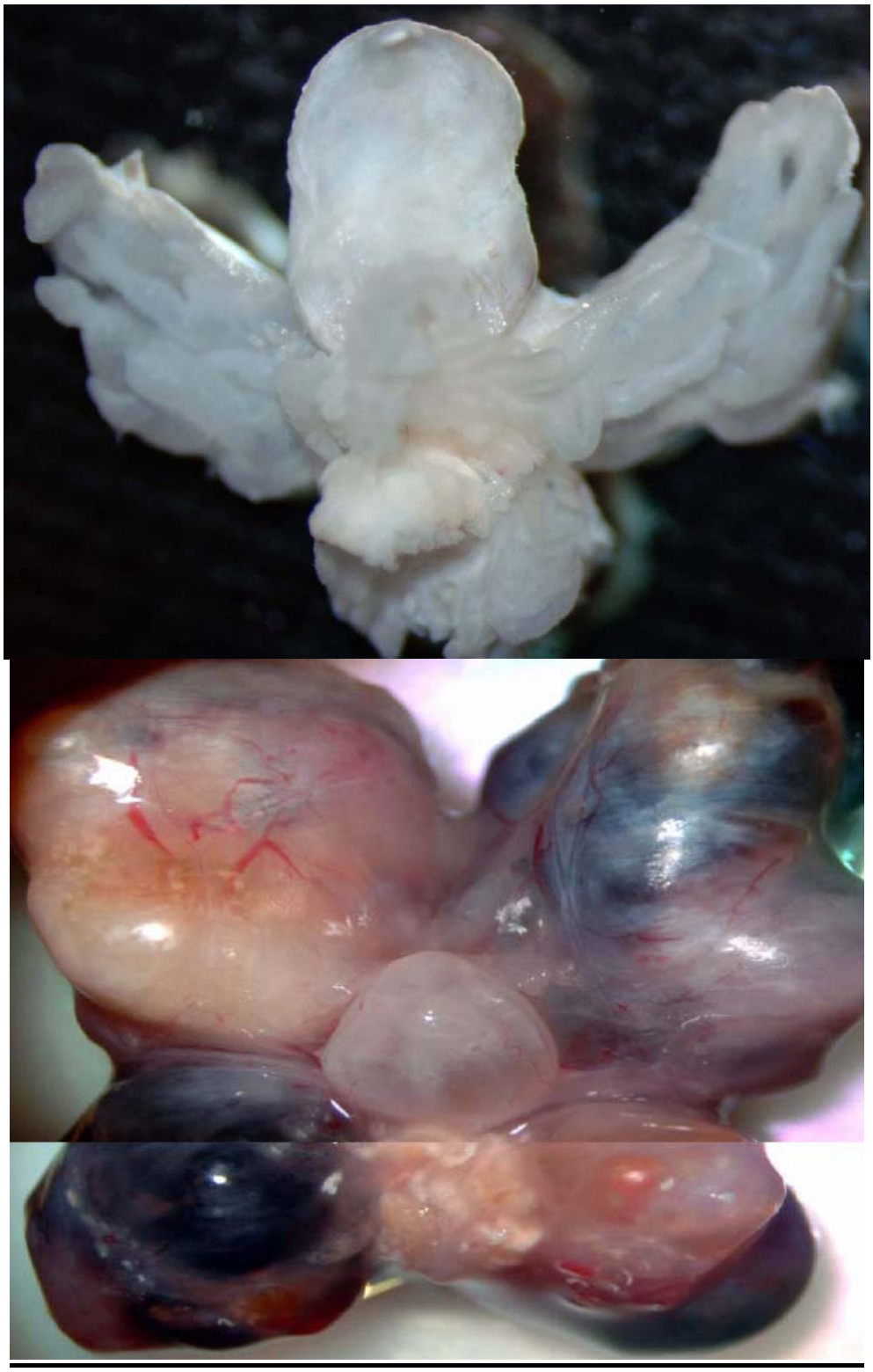

Figure 7. Prostate-specific loss of both *Apc* and *Pten* induces tumorigenesis in a synergistic manner. Shown are whole mount images of prostates dissected from wild type (top) and *PB-cre;Apc-flox/flox;Pten-flox/flox* five month old males. Note the extensive overgrowth and discoloration of the mutant prostate.

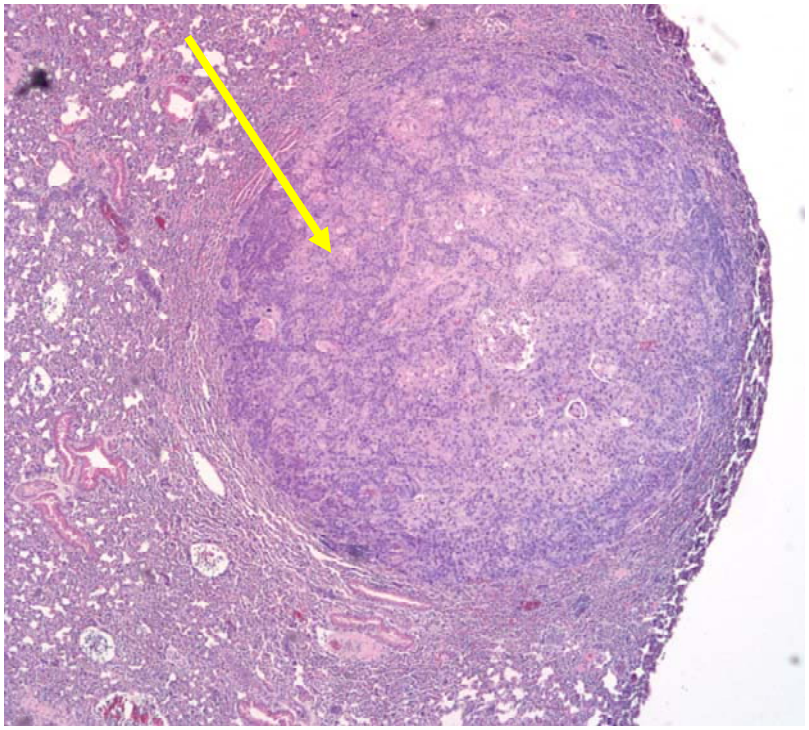

Figure 8. *PB-cre;Apc-flox/flox* mice carrying a prostate specific activatable oncogenic *K-ras* allele develop metastatic tumors by 5 months of age (yellow arrow denotes tumor metastasis in lung**.**

6. Key Research Accomplishments

We have produced what, in my opinion, are several key research accomplishments. These are described in the previous section and can be summarized as the following:

1. We have created and completed initial characterization of strains of mice that expresses the TetON protein specifically in the prostate. These strains (PB1764 and PB1655) will not only allow us to progress in our experiments for the completion of the work proposed for this grant, but should also be an important resource for others in the field of prostate cancer to conditionally express other genes in a prostate-specific manner.

2. During our characterization of the PB1764 strain, we showed that ectopic expression of an activated K-ras gene leads to the development of prostatic dysplasia. This result was obtained by doing a small pilot control experiment for the work to eventually dysregulate the Wnt signaling pathway in the prostate, but is interesting in and of itself. This is due to the fact that several publications have linked ras activation to some prostate cancers. Perhaps most interesting is that ras mutations are associated more commonly with prostate cancer in Japanese populations relative to American populations (25). Thus, further work on this observation may reveal insights into genetic background differences and the development of prostate cancer.

3. We have also developed a mouse model for dysregulated Wnt signaling in the prostate. In this model, loss of *Apc* leads to the development of early onset prostate cancer. Furthermore, this model appears to develop a metastatic version of the disease upon aging.

4. We have demonstrated that that prostate-specific deletion of *Apc* leads to prostate cancer that remains androgen-dependent for growth. We are currently aging castrated males to determine whether these tumors are ever capable of recurring in an androgen-independent manner. Our initial analysis has found that small androgen-independent lesions are indeed capable of forming.

6. We have preliminary evidence that loss of both *Apc* and *Pten* leads to a significant enhancement of prostate tumorigenesis and metastatic progression.

7. We also have preliminary evidence that loss of *Apc* and activation of *K-ras* leads to early onset metastatic prostate carcinoma

7. Reportable Outcomes

A. Abstracts (see attached information in the Appendix)

B. Presentations

The abstracts included in the Appendix were all submitted as poster presentations for the indicated meetings. In addition, I also presented an oral presentation at the Michigan Prostate Colloquim Meeting on May 1, 2004. A summary of events at which this work (or portions of this work) was presented in the past twelve months is included below.

We are also currently preparing a manuscript on this work for submission to a peer-reviewed publication (we have been waiting for the completion of the androgen-deprivation experiments before submitting).

Oral Presentations

1. Michigan Prostate Colloquim Meeting, May 2004.
2. NIDCR Seminar Series, National Institutes of Health, Bethesda, MD, April 2005.
3. Karmanos Cancer Center Retreat, Detroit, MI, October 2005
4. Wayne State Department of Pathology Seminar Series, Detroit, MI, February 2006

Poster Presentations

1. Wnt Meeting, Ann Arbor, Michigan, May 2004
2. AACR Pathobiology of Cancer Meeting, Snowmass, Colorado, July 2004
3. AACR Annual Meeting, Anaheim, California, April 2005
4. British Society of Developmental Biology Wnt Signaling Meeting, Aberdeen, Scotland, September 2005
5. Tucson Symposium, Tucson, Arizona, April 2006

C. Animal Models

As can be determined by reading the body of this report, the vast majority of work in support of this grant award is focused on the generation of mouse models for human prostate cancer. A summary of these is provided below in this section.

1. We have created a mouse in which the TetON protein is expressed specifically in the prostate. This mouse strain is useful for not only some of our studies outlined in this report, but should also be useful to the field in generally as it allows for prostate specific expression of any transgene that is controlled by a tetracycline responsive element.

2. In a pilot study to determine the functionality of the Probasin-TetON strain, we have shown that mice that express activated K-ras specifically in the prostate develop prostatic dysplasia.

3. We have also created mice that lack the Apc gene specifically in the prostate and shown that they develop early onset prostate cancer which progresses to a metastatic state.
4. Other models of Wnt signaling dysregulation in the prostate are currently being developed based on the strains outlined above.

5. In preliminary work, we have found that loss of both Apc and Pten leads to significantly enhanced prostate tumorigenesis.

6. Simultaneous inactivation of Apc and activation of K-ras appears to induce (at least in preliminary experiments) early onset metastatic prostate cancer.

8. Conclusions

We can already conclude based on our mouse models that alterations in the Wnt signaling pathway lead to early onset prostate cancer. We have found that these tumors develop in a very consistent pattern of progression and occur in all *PB-cre;Apc-flox/flox* mice examined. Also, our work indicates a substantial synergistic effect between *Apc* and *Pten (*or *K-ras)* mutations in the development of prostate cancer.

In terms of the "so-what" factor, we believe these observations important as a scientific products. In the past, human prostate tumors had been shown to contain mutations in genes of the Wnt signaling pathway. However, it was not clear if these mutations had anything to do with the intiation or progression of the tumor. Two other recent reports have suggested that alterations in the Wnt pathway can induce changes in the prostate (26, 27). However, we have created a model system in which invasive prostate tumors develop as a result of inactivating *Apc*, representing the first demonstration of induction of invasive prostate cancer by dysregulation of the Wnt pathway. We believe this observation has important implications in examining patients with human prostate cancer and in developing treatments to inhibit progression of the disease. Our ongoing work on synergism between *Apc* and *Pten* mutations (and *Apc* and *K-ras* mutations) in the prostate also has the potential to provide important insights into the progression of prostate cancer and its metastatic progression.

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